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Contrasting responses of stomatal conductance and photosynthetic capacity to warming and elevated CO_2 in the tropical tree species *Alchornea glandulosa* under heatwave conditions

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Abstract

Factorial experiments of combined warming and elevated CO_2 are rarely performed but essential for our understanding of plant physiological responses to climate change. Studies of tropical species are particularly lacking, hence we grew juvenile trees of *Alchornea glandulosa* under conditions of elevated temperature (+1.5°C, eT) and elevated CO_2 (+400ppm, eC) in a factorial open top chamber experiment. We addressed three questions: i) To what extent does stomatal conductance (g_s) reduce with eT and eC treatments?; ii) Is there an interactive effect of eT and eC on g_s?; iii) Does reduced g_s as a result of eT and/or eC cause an increase in leaf temperature?; iv) Do the photosynthetic temperature optima (T_{opt}) and temperature response of

photosynthetic capacities (V_{cmax}, J_{max}) shift with higher growth temperatures? The experiment was performed during an anomalously hot period, including a heatwave during the acclimation period. Our key findings are that: 1) the eT treatment reduced g_s more than the eC treatment, 2) reduced g_s caused an increase in leaf temperatures, and 3) net photosynthesis and photosynthetic capacities showed very high temperature tolerances with no evidence for acclimation to the eT treatment. Our results suggest that *A. glandulosa* may be able to cope with increases in air temperatures, however reductions in g_s may cause higher leaf temperatures beyond those induced by an air temperature rise over the coming century.

Keywords: photosynthesis, climate change, factorial experiment, tropical forest, warming, carbon dioxide, leaf temperature, vcmax, jmax, temperature optima, open top chamber, photosynthetic capacity

1. Introduction

Global atmospheric CO_2 concentrations are increasing, as are air temperatures, with both patterns expected to continue in the coming decades. Plants are a critical part of global biogeochemical cycles, at the interface of the atmosphere and the land surface, with forests storing 65% of terrestrial aboveground biomass (Liu et al., 2015). Plants respond to environmental stimuli, with long-term adaptation and short-term acclimation to changes in light, temperature and other conditions. Photosynthesis, evapotranspiration, and respiration are the primary functions of leaves. Our understanding of leaf-level physiology is used to drive vegetation and land surface models, and hence to project future climate. Experimental research on the responses

of forests to elevated CO_2 has been heavily focussed on temperate ecosystems (Leakey et al. 2012) despite tropical forests stocking more carbon than temperate and boreal forests combined (Pan et al. 2011). Similarly, there are very few studies of thermal acclimation on tropical species (Dusenge & Way 2017). Although temperature increases in the tropics are predicted to be smaller than in other regions (e.g. boreal zone, Collins et al. 2013), tropical forests experience much lower diurnal and seasonal variation in temperature than temperate or boreal forests, and over geological time have experienced a relatively stable climate, potentially reducing the acclimation potential of tropical tree species (Janzen 1967, Dusenge & Way 2017). Investigating the responses of tropical tree species to temperature and CO_2 is therefore a research priority.

Increasing air temperatures and atmospheric CO_2 concentrations lead to changes in stomatal conductance (g_s) over short and long timescales (Way et al., 2015). In the short-term (instantaneous responses), increasing air temperatures typically lead to a reduction in g_s (Way et al., 2015, Slot & Winter 2017a) due to stomatal closure with increasing vapour pressure deficit (D), which prevents excessive water loss under high evaporative demand. At very high temperatures, g_s may actually increase in order to avoid reaching dangerously high leaf temperatures (Slot et al., 2016, Slot & Winter 2017b, Urban et al., 2017, Drake et al., 2018). Evidence of acclimation of g_s to higher temperatures in trees over the long-term is varied, however some species show declines (Way et al., 2015). The instantaneous response of g_s to increased CO_2 is to decrease, which reduces water loss while maintaining a high internal leaf CO_2 concentration (c_i) (Gaastra, 1959). Similarly, under long-term CO_2 enrichment, g_s reduces. Such declines in g_s may increase leaf temperature (T_L) through reduced

76 evaporative cooling (under increased air temperatures, reduced evaporative cooling
77 would also depend on the extent of increased D, Oren et al. 1999). Higher T_L could
78 push leaves beyond their photosynthetic temperature optima (T_{opt}) (Doughty &
79 Goulden 2008, Slot & Winter 2017c), and potentially above their physiological
80 temperature tolerances (O'Sullivan et al., 2017) causing permanent leaf damage under
81 extreme heat conditions (Warren et al., 2011). While the response of g_s to combined
82 elevated CO_2 (eC) and temperature (eT) has rarely been tested (Way et al., 2015,
83 Becklin et al., 2017), experiments on eucalyptus (Ghannoum et al., 2010), douglas-fir
84 (Lewis et al., 2002) and loblolly pine (Wertin et al. 2010) showed little interactive
85 effect; if the two do interact and lead to even greater decreases in g_s, this would
86 increase T_L further.
87
88 Long-term increasing air temperatures and CO_2 concentrations are also predicted to
89 induce changes in net photosynthesis, both directly by impacting biochemical
90 processes and indirectly through changes in g_s. Increases in T_L either directly from
91 increased air temperatures or indirectly from a long-term reduction in g_s could shift
92 the leaf beyond T_{opt}, leading to reductions in photosynthesis. Some experimental
93 studies have shown partial photosynthetic acclimation to increasing temperatures
94 through increases in T_{opt} (Yamori et al., 2014, Slot & Winter 2017b), which could
95 occur due to alterations in membrane fluidity, expression of heat shock proteins, and
96 production of greater quantities of Rubisco activase or a heat-stable Rubisco activase
97 (Yamori et al. 2014). These changes would lead to altered temperature responses of
98 the photosynthetic capacities V_{cmax} (maximum rate of carboxylation) and J_{max}
99 (maximum rate of electron transport). A recent study of four tropical tree species
100 showed that g_s rather than V_{cmax} or J_{max} limited net photosynthesis beyond T_{opt} (Slot &

Winter 2017a), and hence a change to the temperature (or D) response of g_s could also be important for shifts in T_{opt}. Photosynthetic capacities are also influenced by growth CO_2 concentrations. Under high CO_2, Rubisco concentrations typically reduce and hence V_{cmax} declines (Way et al. 2015). Decreases in g_s (as a consequence of increased air temperature or CO_2) lead to reduced c_i which can reduce assimilation. Under high CO_2 concentrations, this effect could be limited if c_i remains above the Rubisco limited portion of the A-c_i curve, however the downregulation of V_{cmax} commonly observed results in plants still being Rubisco limited even at high CO_2 (Ainsworth & Rogers 2007) and hence reduced g_s could still reduce assimilation (Way et al., 2015).

The effect of decreased conductance on T_L is well understood biophysically (Jones 1992) and is expected to influence T_L under elevated CO_2 (Drake et al. 1997), as has been shown in a small number of experiments (e.g. Siebke et al. 2002, Sigut et al. 2015). However, this effect has not been investigated in any tropical species. Furthermore, because T_L and, to a lesser extent, g_s show high temporal variation with changing microclimate (e.g. Fauset et al., 2018), to fully investigate the effect of altered g_s as a response to elevated temperature and CO_2 it is necessary to measure T_L and microclimate with a high temporal resolution.

In this study, we address the following questions using a factorial eT x eC open top chamber experiment with juveniles of tropical tree species *Alchornea glandulosa* (Poepp. & Endl) (Euphorbiaceae): i) To what extent does g_s reduce with elevated temperature (eT) and elevated CO_2 (eC) treatments?; ii) Is there an interactive effect of eT and eC on g_s?; iii) Does reduced g_s as a result of eT and/or eC cause an increase in T_L?; iv) Do the photosynthetic temperature optima (T_{opt}) and temperature response

126 of photosynthetic capacities (V_{cmax}, J_{max}) shift with higher leaf temperatures? *A.*
127 *glandulosa* is a pioneer species often found, but not restricted to, riverine
128 environments (Pascotto 2006), distributed in the Atlantic forest, western
129 Amazon/Andes and central America (GBIF Secretariat 2017), with over 100,000,000
130 individual trees estimated to occur in the Amazon (ter Steege et al., 2013). It is
131 utilized as a timber species, produces medicinal compounds and is used for
132 reforestation in the Atlantic forest region. The fruits of this tree are an important food
133 source for birds (Pascotto 2006). This species was also selected because leaf
134 temperature and stomatal conductance field data for congeneric species *Alchornea*
135 *triplinervia* were available from the Atlantic forest (Fauset et al., 2018).
136
137 **2. Methods**
138
139 *2.1 Experimental setup*
140
141 The study was carried out at the University of São Paulo from February to March
142 2017 (23.56° S, 46.73° W, elevation 760 m). *Alchornea glandulosa* seedlings were
143 sourced from a local plant nursery where they were germinated in shade houses
144 before growing for 12 months outside.
145
146 The seedlings were moved to the glasshouse in September 2016 and in November
147 transferred into containers (4*l* PVC pots with one plant per plot). Hoagland fertilizer
148 solution was added every 2 weeks. The experiment was conducted using four
149 polycarbonate open top chambers (OTCs) with modifications (Aidar et al., 2002)
150 located within the glass house. The four treatments were: i) control (aTaC), ii)

151 elevated CO_2 (ambient temperature, 800 ppm CO_2, aTeC), iii) elevated temperature
152 (temperature 1.5°C above ambient, ambient CO_2, eTaC), and iv) elevated CO_2 and
153 elevated temperature (temperature 1.5°C above ambient, 800 ppm CO_2, eTeC). Each
154 chamber had an air inlet at the base with a fan, and a spiral heater and/or CO_2 gas inlet
155 was present depending on the treatment (Figure S1). Temperature within the chamber
156 was thermostatically controlled using RICS software (Remote Integrated Control
157 System) with the heater switched on or off to maintain a higher temperature than the
158 unheated chambers. No attempt was made to control for differences in *D* due to
159 temperature treatments as increases in temperature would be associated with increases
160 in *D* under future conditions assuming no change in relative humidity. CO_2 was
161 passively added to the eC treatments through the use of pressurized CO_2 cylinders.
162 The CO_2 concentrations of the eC chambers was monitored daily and the flow into the
163 chambers altered at a valve if the concentration decreased. Further details of the
164 experimental design can be found in Aidar et al., 2002 and de Souza et al. (2008). Ten
165 seedlings were placed into each chamber on 1 February 2017 and allowed to
166 acclimate for one month before measurements began. Vertical height of each seedling
167 was recorded prior to placement in the OTCs, and placement of seedlings into OTCs
168 was stratified to ensure an even spread of vertical heights.
169
170 *2.2 Microclimate measurements*
171
172 Within each OTC air temperature (T_A), relative humidity (h) and CO_2 concentration
173 were measured at 5 min intervals (Testo 535, Testo Inc., Flanders, NJ, USA). An
174 additional T_A sensor (107 thermistor, Campbell Scientific) recorded air temperature
175 every 10 s inside each chamber.

176

177 *2.3 Physiological Measurements*

178

179 2.3.1 Leaf temperature and leaf surface PAR

180 The eight healthiest of the ten seedlings in each chamber were selected for
181 measurement of leaf temperature. On each selected seedling, one fully expanded
182 healthy leaf was chosen (typically the fourth or fifth newest leaf). These leaves were
183 formed inside the glass house but prior to movement of the seedling into the OTCs.
184 Prior to selection, we verified that the leaves were photosynthetically active. A two-
185 junction thermocouple (copper-constantan, type T) that measured leaf-to-air
186 temperature difference (ΔT_L) was attached to the abaxial surface of each sample leaf
187 using a piece of breathable tape (Transpore, 3M, St. Paul MN) following the protocol
188 of Fauset et al. (2018). One thermocouple was used per leaf. Absolute leaf
189 temperatures (T_L) were calculated from ΔT_L and T_A in each chamber measured by the
190 thermistors. A photosynthetically active radiation (PAR) sensor built to the
191 specification of Fielder & Comeau (2000) was positioned adjacent to each sample leaf
192 at the same angle and orientation. PAR sensors were calibrated against a quantum
193 sensor (LightScout, Spectrum Technologies, Aurora, Illinois). ΔT_L and leaf surface
194 PAR were monitored continuously at 10 s measuring frequency between 24 February
195 – 15 March 2017 using two CR800 data loggers and two AM16/32 multiplexers
196 (Campbell Scientific). Measurements of some leaves were terminated between 10 and
197 15 March. See Fauset et al. (2017, 2018) for further details of these sensors.

198

199 2.3.2 Stomatal Conductance

Stomatal conductance (g_s) of each leaf temperature sample leaf was measured under growth conditions inside the chambers on 19 occasions over six days (including four days where g_s of each leaf was measured at least four times, 28 February – 7 March 2017) using an SC-1 porometer (Decagon). For each time point, two measurements of g_s were recorded, one from either side of the midrib, and the mean value was used for analysis.

2.3.3 Photosynthetic measurements

The temperature response of photosynthesis was measured using a LI-COR 6400XT portable photosynthesis measurement system (LI-COR, Nebraska). Data were collected from 10 – 18 March 2018. Light response curves on 3 leaves showed saturating photosynthesis at 800 μmol m^{-2} s^{-1} PAR (Figure S2), hence all measurements were taken at 800 μmol m^{-2} s^{-1} PAR using the standard red-blue LED light source. Note that the glasshouse roof was made of a diffusing plastic which reduced the incoming PAR by c. 60 % compared with the outside, and leaf level PAR reached c. 800 μmol m^{-2} s^{-1}, varying with leaf angle and orientation. Three seedlings from each OTC were selected for photosynthesis measurements and the leaf measurements were performed on the same leaf as leaf temperature monitoring. Two sets of measurements were made, net photosynthesis-temperature curves (A-T_L curves where net photosynthesis at saturating light intensity is measured at different temperatures), and A-c_i curves (where net photosynthesis at saturating light intensity is measured at different CO_2 concentrations) at three different temperatures. A-T curves were run with the CO_2 concentration of the relevant OTC (either 400 of 800 ppm CO_2) and assimilation was measured at leaf chamber temperatures of 20, 25, 27, 29, 31, 33, 35 and 40 °C, with 5 measurements recorded at each temperature after the

photosynthetic rate and g_s had stabilized. Measurements at 20, 30 and 35 °C were supplemented using the relevant measurements from the A-c_i curves. A-c_i curves used the following sequence of CO_2 concentrations (ppm); 400, 200, 100, 50, 400, 600, 800, 1200, 1500, 2000. A-c_i curves were performed at three temperatures, 20, 30, and 35 °C, and each curve was performed twice for each leaf on either side of the midrib. For all measurements, h was maintained as close as possible to 50 % using a combination of desiccant and adjusting the air flow rate; it was difficult to maintain this h at leaf temperatures above 37 °C (on average 46 %, minimum values were 40 %). The temperature of the chamber was mostly controlled using the inbuilt temperature control system. In addition, for most of the measurements the sensor head was placed inside a specially designed temperature control chamber to enable better control of the chamber temperature (Yepes Mayorga 2010). The temperature control box was switched off during measurements but was used to aid the change of chamber temperature between measurements. Measurements were made at an atmospheric pressure in the greenhouse of 92.6 kPa.

2.3.4 Plant growth

Vertical height (from soil surface) and number of leaves of each seedling was measured three times (1 and 21 February, and 16 March). On the latter two measurement days, the length of the seedling from the soil surface to the end of the longest branch was also recorded, and on 16 March the total plant length including all branches was recorded.

2.4 Data analysis

Differences in microclimate between OTCs (air temperature, CO_2 concentration, h and D) were tested using ANOVA and Tukey post-hoc test.

The effects of the warming and the elevated CO_2 treatments on g_s (porometer measurements pooled from all times of day) were tested using two-way ANOVA with a mixed effects model with leaf as a random factor to account for multiple measurements of the same leaves (function 'lme' of the R package nlme, Pinheiro et al. 2017). To investigate the response of g_s to microclimate variables and under different treatments, all possible models of PAR and leaf-to-air vapour pressure deficit D_L (where leaf temperature was taken from thermocouple data), with interactions with CO_2 treatment and warming treatment were compared using AIC to select the best model with the function 'dredge' in R package MuMIn (Bartoń 2017). Again, a linear mixed effect model with leaf as a random factor was used to account for multiple measurements of the same leaf/seedling. A quadratic effect of time was also included in the model to account for diurnal changes in g_s not directly linked to PAR, temperature or D_L. R^2 for mixed-effects models are given using as the marginal pseudo R^2 that accounts for fixed factors only rather than the conditional pseudo R^2 which also accounts for random effects (Nakagawa & Schielzeth 2013) unless otherwise stated; R^2 values for mixed effects models were calculated using the function provided in the R package MuMIn. We also estimated the g_1 parameter of the optimal stomatal conductance model (Medlyn et al. 2011, Lin et al. 2015) from the A-T_L curve data collected with the LI-COR 6400.

$$g_s = 1.6\left(1 + \frac{g_1}{\sqrt{D}}\right)\frac{A}{C_a}$$

where C_a is the atmospheric CO_2 concentration in the leaf chamber. The model was fit for each leaf, and the g_1 parameter was compared between chambers using ANOVA.

Because leaf temperatures are strongly influenced by microclimate (Jones 1993, Fauset et al. 2018), to assess the influence of treatment on T_L it is necessary to compare T_L within microclimatic envelopes. We subsetted the data into envelopes based on leaf-level PAR, chamber air temperature and *D*. The data was split into low (100 – 200 μmol m^{-2} s^{-1}), medium (400 – 500 μmol m^{-2} s^{-1}) and high (700 – 800 μmol m^{-2} s^{-1}) PAR, and low (28 – 30 °C, 1 - 2 kPa), medium (33 – 35 °C, 2 - 3 kPa), and high (38 – 40 °C, 3 - 4 kPa) air temperature and *D*. An unanticipated effect of the switching on and off of the heater in the warmed chambers was a cycle in leaf temperature. This was particularly clear at night, but also occurred during the day. When the heater was switched on, the ΔT_L became more negative as the air heated faster than the leaf (Figure S3). The ΔT_L then rose to reach an equilibrium temperature. Because of this cycle in the ΔT_L data, it was not possible to compare leaf temperatures directly between the ambient and heated chambers, and hence direct comparisons on ΔT_L were only made between CO_2 treatments within temperature treatments.

The temperature response of photosynthesis is typically modelled as a parabolic curve which provides a T_{opt} parameter (e.g. Robakowski et al. 2012). However, as no evidence of a decline of *A* with increasing T_L was found (see section 3.4), we could not use the parabolic curve to find T_{opt} (Fig. S4) which was beyond the range of our measurements. Hence, a linear mixed effect model with leaf as a random factor was used to test the relationship between *A* and T_L. As for stomatal conductance we selected the best model based on AIC from all possible models, here including T_L as a continuous fixed effect and interactions with CO_2 treatment and warming treatment.

V_{cmax} and J_{max} were estimated for each leaf and each temperature from the A-c_i curve using the Farquhar-von Caemmerer-Berry model using the R package plantecophys (Duursma 2015). For some curves (six for J_{max} and one for V_{cmax}, all at 20 °C), the parameters could not be adequately estimated and estimates were not used. Of the remaining fits, the root mean square error ranged 0.18 – 1.57 μmol m^{-2} s^{-1}. The temperature responses of V_{cmax} and J_{max} were modelled using the Arrhenius function (Medlyn et al. 2002)

$$f(T_k) = k_{25} \cdot exp\left(\frac{E_a(T_k - 298)}{(298RT_k)}\right)$$

where k_{25} is the value of V_{cmax} or J_{max} at 25 °C, E_a is the activation energy (kJ mol^{-1}), T_k is the leaf temperature (°K) and R is the universal gas constant (8.314 J mol^{-1} K^{-1}) . The parameters were fit using non-linear least squares (R function nls). This function was fit separately for each chamber, and significant differences in parameter estimates were tested by comparing the 95 % confidence intervals (following Varhammar et al. 2015). A peaked Arrhenius function was not used as the data did not show a decline in V_{cmax} or J_{max} at high temperatures.

3. Results

3.1 Microclimate over the study period

The experimental period coincided with an anomalously hot summer in São Paulo city including a 4 day heatwave (Figure 1). Using the definition of a heatwave from

Russo et al. (2015) as ≥3 consecutive days where the maximum temperature exceeds the 90th percentile of maximum temperatures from a monthly window for the period 1981-2010, and climate data for the Mirante de Santana weather station (INMET), a four day heatwave period occurred (maximum temperatures above 32.3 °C) in mid-February (Figure 1, Figure S5). The heatwave occurred during the acclimation period but before the initiation of data collection. During this time the maximum daily air temperatures within the OTCs exceeded 45 °C (Figure 1).

Mean daily temperatures within the OTCs over the acclimation and measurement periods were significantly different between chambers (F = 5.4, P = 0.001, ANOVA, Figure 2a). Temperatures were significantly lower in the aTeC treatment (28.8 ± 2.3 °C mean ± SD) than the eTeC treatment (30.4 ± 2.3 °C), however the difference between aTaC (29.0 ± 2.2 °C) and eTaC (30.3 ± 2.4 °C) was marginally insignificant (P = 0.07, Tukey post-hoc test, Figure 1a). Mean daily CO_2 concentration was significantly higher in the aTeC and eTeC treatments (829.9 ± 71.6 ppm and 836.7 ± 70.6 ppm, respectively, Figure 1b) than the ambient CO_2 treatments, however the concentration in the eTaC chamber (399.0 ± 8.9 ppm) was significantly lower than the aTaC chamber (459.2 ± 12.2 ppm). Relative humidity also varied by treatment with lower values in the elevated CO_2 treatments (Figure 2c), and D was higher in elevated temperature treatments, significantly so for eTeC (Figure 2d).

3.2 Stomatal Conductance

Analysing g_s data with measurements at all times of day pooled, g_s was significantly lower under the elevated temperature treatments (P = 0.0001, mixed effects model

with leaf as a random factor), with no significant effect of CO_2 treatment (Figure 3). Conductance was highest in the control treatment and similarly low in both elevated temperature treatments, with an intermediate g_s in the aTeC treatment (Figure 3).

The best mixed effects model of g_s accounting for microclimate and diurnal changes included time of day, PAR, D_L, and interactions between D_L, warming treatment and CO_2 treatment (Figure 4, Table 1). The overall pseudo marginal R^2 of the model was 0.38. If the random effect of leaf is also accounted for, the pseudo conditional R^2 increases to 0.67 showing that there is high leaf-to-leaf variation in g_s (Supplementary Figure 6). Interaction plots (Figure 4) of the model show that the relationship between g_s and D_L was weak (with no significant effect of D_L alone, Table 1) and varied between treatments (interactions between heat treatment and D_L, and heat treatment, CO_2 treatment and D_L were significant, Table 1). Under the aTeC and eTeC treatments g_s was fairly invariant with D_L, whilst under the eTaC treatment g_s declined with D_L and under the control aTaC treatment g_s increased with D_L. However, there is large scatter in the data (Figure 4, Figure S6).

The parameter g_1 (inversely proportional with the carbon cost of transpiration and hence low when a plant is conservative in its water use) estimated from the A-T_L curves did not show any significant differences between chambers, despite a lower mean for the eTaC chamber (Figure 5).

3.3 Observed Leaf Temperatures

374 Diurnal patterns of average ΔT_L, T_L, PAR and D are shown for all chambers in Figure
375 6 based on the period 24 February – 15 March 2017. There are differences in the
376 patterns of average ΔT_L for each chamber (Figure 6c,d), and these patterns are linked
377 to the patterns of average PAR (Figure 6e,f). In order to properly compare the leaf
378 temperatures between different leaves and chambers, the varying microclimate needs
379 to be accounted for.
380
381 Mean ΔT_L values were not significantly different between elevated and ambient CO_2
382 within the warming treatment under any specified microclimate (Figure 7, eTeC
383 versus eTaC). In contrast, under the majority of microclimates tested ΔT_L values were
384 significantly higher in the elevated CO_2 treatment compared to the ambient CO_2
385 treatment when under ambient temperatures (Figure 7, aTeC versus aTaC). The
386 microclimate conditions under which no significant differences were found were both
387 in the high PAR category where there were much fewer data points, and the pattern in
388 the data was similar to other microclimates. The extent of the difference in ΔT_L
389 between aTaC and aTeC increased under increasing air temperature and increasing
390 PAR, with a difference of 2.8 °C under high PAR and high air temperature. Analysing
391 the data for T_L rather than ΔT_L produced the same results (data not shown).
392
393 *3.4 Photosynthetic Temperature Response Curves*
394
395 Despite measuring photosynthesis at leaf temperatures up to 40 °C, there was no
396 evidence of reaching T_{opt} as A continued to increase with T_L for the majority of leaves
397 (Figure 8). Consequently, estimation of T_{opt} was not attempted and linear models were
398 used to analyse the A-T_L curves. There was no significant effect of temperature

399 treatment, however $T_{L,}$ CO_2 treatment and their interaction were included in the best
400 model. *A* (measured at the growth CO_2 concentration) was higher and the slope of the
401 *A-T* relationship was steeper under the elevated CO_2 treatments (Figure 8). The
402 marginal pseudo-R^2 of the model was 0.53, and all model terms (T_L, CO_2 treatment
403 and their interaction) were significant (Table 2).
404
405 *3.5 Temperature Responses of V_{cmax} and J_{max}*
406
407 As for *A*, both V_{cmax} and J_{max} increased with measurement temperature (Figure 9) and
408 no optimum temperature was found within the measurement range (20 – 35 °C).
409 V_{cmax} varied ranged 6.1 – 51.8 μmol m^{-2} s^{-1} and J_{max} ranged 16.3 – 46.3 μmol m^{-2} s^{-1}
410 with standard errors ranging 0.097 – 6.88 μmol m^{-2} s^{-1} for V_{cmax} and 0.29 - 2.76 μmol
411 m^{-2} s^{-1} for J_{max}. The higher SE values correspond with higher parameter values.
412 Temperature treatment had no significant effect on either of the two variables,
413 however V_{cmax} was lower and the temperature response of V_{cmax} was weaker (lower
414 activation energy) under elevated CO_2, with significant differences between eTaC and
415 eTeC treatments (Figure 9, Table 3). The ratio of J_{max}/V_{cmax} decreased with increasing
416 temperature (30 - 35 °C, not sufficient J_{max} data at 20°C), and was significantly higher
417 in the elevated CO_2 treatment (Figure S7).
418
419 *3.6 Seedling growth*
420
421 There were no significant effects of treatment on seedling size at any time point
422 during the experiment (vertical height, total branch length, number of leaves, Figure
423 S8).

4. Discussion

In this study we present a factorial elevated temperature and elevated CO_2 experiment with juveniles of a tropical pioneer species. The study was performed under high temperature conditions including a heatwave during the acclimation period (Figure 1). Our key findings are i) that the elevated temperature treatment had a stronger influence on g_s than elevated CO_2 (Figure 3, Table 1), ii) that reduced g_s caused a change in leaf temperatures (Figure 7), iii) that net photosynthesis and photosynthetic capacities show very high temperature tolerances with no evidence for acclimation to the elevated temperature treatment (Figure 8), and iv) that there was no interactive effect of temperature and CO_2 treatment on g_s (Figure 3, Table 1)

4.1 Temperature and CO_2 impacts on stomatal conductance

As expected, g_s declined in the eC treatments compared with the control, as has been shown in many other studies. Here we find a 21.2 % reduction (95% CI 10.6 – 30.2 % based on bootstrapping) in our aTeC treatment compared with the control (Figure 2). In forest free air CO_2 enrichment (FACE) experiments with CO_2 elevated by 200 ppm g_s declines on average by *c*. 20 % (Ainsworth & Rogers 2007), with stronger declines in angiosperm than gymnosperm species (Brodribb et al. 2009). Past chamber experiments performed on angiosperm trees with a doubling of CO_2 show an average g_s reduction of *c*. 18 % (from data in Saxe et al. 1998). Our data therefore shows consistency with species from other biomes, but with few tropical species included in existing studies. The literature on tropical species shows wide variation (Berryman et

449 al. 1994, Goodfellow et al. 1997, Liang et al. 2001, Leakey et al. 2002, Khurana &
450 Singh 2004, Cernusak et al. 2011, Dalling et al. 2016, Wahidah et al. 2017). Data
451 from eight publications covering 22 tropical angiosperm species with CO_2 enrichment
452 in the range 300-400 ppm showed an average change in g_s of 28.6 ± 18.4 % SD
453 reduction. One species (*Chrysophyllum cainito*) showed a very small increase
454 (Dalling et al. 2016), and the largest reduction of 61 % was shown by *Inga punctata*
455 (Cernusak et al. 2011). Hence, the reduction we observed was below average but well
456 within the range of observations of other tropical species in experiments.
457
458 A limitation of our experiment and its comparability with other studies is the short
459 duration of exposure to the treatments. We measured the physiological responses on
460 leaves formed before initiation of the experiment, which had been exposed to the
461 treatments for *c*. 5 weeks. As stomatal properties (e.g. density) often differ on leaves
462 formed in high CO_2 environments (Saxe et al. 1998), there could potentially be
463 greater changes than we observed, had new leaves formed. Whilst this is quite
464 possible, the long-term response of g_s to CO_2 is typically similar to the short term
465 response (Way et al. 2015), and hence while the mechanism of reduced g_s may be
466 different in short and long-term studies, the g_s may be similar. However, a caveat to
467 our results is that to truly observe the acclimation of leaves to the treatments, longer
468 acclimation periods and production of new leaves is necessary.
469
470 The observed responses of g_s to elevated temperature vary considerably in the few
471 studies available (Way et al. 2015). Here we find strong reductions in g_s in the
472 temperature treatments with a 49.6 % (95% CI 42.2 – 56.5 %) reduction under the
473 eTaC treatment and 53.0 % (95% CI 52.9 – 58.3 %) reduction in the combined eTeC

474 treatment, although we did not find any significant difference between treatments for
475 the g_1 parameter value. This may be because the Medlyn et al. (2011) model
476 incorporates the ambient CO_2 concentration, and if the short-term and long term g_s
477 response to CO_2 is the same there would not be a difference. The declines in g_s are not
478 driven purely by higher D_L in the eT chambers as there are significant differences
479 even when D_L is controlled for (Table 1, Figure 4) or when g_s is analysed within a
480 narrow D_L range (data not shown). This shows acclimation of g_s due to higher air
481 temperature and/or D_L (both quantities strongly co-varied) which will reduce water
482 loss from the plants. There were no significant differences in g_s between the eTaC and
483 eTeC treatments, hence the response to the temperature treatment (with significant
484 differences) was stronger than the response to the CO_2 treatment. The result is
485 surprising given the very mixed results in the limited literature on elevated
486 temperature impacts on g_s, and even more so given that in this study the temperature
487 treatment was fairly modest (+1.5 °C) compared to the CO_2 treatment (+ 400 ppm),
488 although the effect of eC on g_s may have been limited by the lack of new leaf
489 development (as stated above). This finding could also be because the ambient
490 temperatures were very hot inside the chambers throughout the experiment and
491 especially during the acclimation phase (Figure 1), which meant that a small increase
492 in air temperature had a large impact, with stomata closing to reduce water loss. An
493 experimental study of gas exchange of *Solanum lycopersicum* (cherry tomato)
494 measured during and following a +14 °C heatwave showed reduced g_s during the
495 heatwave, which remained low when measured 5 days after the heatwave (Duan et al.,
496 2016). Similarly, Duarte et al. (2016) found reduced g_s of *Pseudotsuga menziesii*
497 (Douglas fir) during +12 °C heatwaves which remained when measured one month
498 later. This is somewhat in contrast with recent research suggesting stomata remain

499 open under very high air temperatures for increased evaporative cooling (Slot et al.,
500 2016, Slot & Winter 2017b, Urban et al., 2017, Drake et al, 2018). Responses are
501 likely to be species specific, with an example of a late successional species reducing
502 g_s under heatwave conditions while a pioneer species showed increased g_s (Vargas &
503 Cordero 2013). However these studies are assessing the instantaneous response of g_s
504 to short-term warming rather than the long-term response. A field study reporting the
505 impact of four months of experimentally elevated temperature on g_s of existing leaves
506 showed a *c*. 25 % reduction with 2 °C temperature increase averaged across six
507 tropical species (Doughty 2011), lower than we observed. However, in contrast to our
508 results for *Alchornea glandulosa*, Yepes Mayorga (2010) found that g_s of *Hymenea*
509 *courbaril* was more strongly controlled by elevated CO_2 than elevated temperature in
510 a similar study, as did Ameye et al. (2012) in a study of temperate species Quercus
511 rubra and Pinus taeda in treatments of elevated by 320 ppm and T_A elevated by 3 °C
512 or with heat waves. Two studies of subtropical/temperate *Eucalytpus* spp. found no
513 difference in g_s of under treatments of CO_2 elevated by 240 ppm and T_A elevated by 3
514 °C or 4 °C after 15 and 7 months of acclimation respectively (Quentin et al. 2013,
515 Duan et al. 2018). While more studies are needed to see if there is a general pattern
516 for tropical broadleaf species, the results of this study suggest that there could be
517 larger implications of rising temperature than rising CO_2 for water use of at least
518 some species of tropical tree, and even implications of modest temperature rises such
519 as the ambitious aims of the Paris Agreement (UNFCCC 2015).
520
521 The g_s dataset also showed a weak relationship with respect to D_L, which varied with
522 treatment (Table 1, Figure 3). Other studies with a congeneric species show that g_s of
523 *A. triplinervia* is more weakly linked to D_L than other measured species (García-

524 Núñez et al. 1995, Fauset et al. 2018). A weaker relationship between g_s and D_L is
525 expected for low wood density pioneer species compared to species with higher wood
526 density (Lin et al. 2015). In addition, as the species is commonly found in riparian
527 areas (and therefore with access to a good water supply), its lack of stomatal control is
528 not surprising. Our results show that despite a weak instantaneous response of g_s to
529 microclimate, *A. glandulosa* still showed acclimation and reduction in g_s in response
530 to long-term microclimate change. Hence, the short-term response of g_s does not
531 provide information on the long-term response.
532
533 4.2 *CO_2 impacts on leaf temperature*
534
535 The lower g_s as a result of elevated CO_2 caused increases in leaf temperatures (Figure
536 5). The differences in ΔT_L increased with increasing PAR at the leaf surface, and to a
537 lesser extent with increasing air temperature and *D*. This shows that the differences in
538 leaf temperatures due to CO_2-altered g_s are more apparent under high thermal stress
539 conditions (high PAR and high air temperature), and therefore that this impact is
540 likely to be stronger under heat waves, which are expected to increase in frequency
541 during the 21st century (Coumou & Robinson 2013). When at high air temperatures,
542 differences in ΔT_L due to reduced g_s could have significant consequences, as seen in
543 observations of premature leaf senescence during a heatwave in a temperate FACE
544 experiment (Warren et al. 2011). While the average differences in ΔT_L between aTaC
545 and aTeC reached 2.8 °C under high light and air temperature, the light conditions
546 were limited by the greenhouse environment which reached only 1000 μmol m^{-2} s^{-1}.
547 Under field conditions where incoming PAR can reach over 2500 μmol m^{-2} s^{-1} the
548 impact of reduced g_s on ΔT_L could be much higher. Unfortunately due to ΔT_L

549 fluctuations induced by heating the air (Figure S3) it was not possible to assess the
550 impact of the high temperature treatment compared to the control. Within the two
551 high temperature treatments there were no significant differences in ΔT_L under any
552 microclimate between the elevated and ambient CO_2 treatments, which is expected as
553 they did not show any significant differences in g_s.
554
555 4.3 *Temperature and CO_2 impacts on photosynthesis*
556
557 The elevated temperature treatment had no discernible effect on A or photosynthetic
558 capacity and their responses to elevated temperatures. The high temperature tolerance
559 of both A and photosynthetic capacity was marked, with no decline in A found even at
560 40 °C. Consequently, we were not able to assess shifts in T_{opt} with treatment as T_{opt}
561 was above the maximum temperature under which we performed measurements. It is
562 worth noting that such high leaf temperatures are often considered to be detrimental to
563 photosynthetic functions (e.g. Rubisco activase activity is strongly temperature
564 sensitive with inhibition found above 35 °C [Crafts-Brandner & Salvucci 2000]).
565 Moreover, photosystem II (PSII) activity declines rapidly above temperature
566 thresholds of 41.5 – 50.8 °C (O'Sullivan et al. 2017). However, plants are well
567 adapted to their environment, with temperature thresholds of PSII increasing from
568 arctic to tropical habitats (O'Sullivan et al. 2017), and even increasing thermal
569 tolerance of PSII over very short timescales (days) in response to high temperatures
570 (Drake et al. 2018). Slot et al. (2017c) found that T_{opt} measured in the field in Panama
571 was around the mean maximum daily temperature (30-32 °C) for all 42 species
572 measured, and that, for a smaller sample of four species, it was g_s rather than Rubisco
573 activase, J_{max}, V_{cmax} or light respiration that limited the photosynthetic rates at high

574 temperatures (Slot & Winter 2017a). In another study, T_{opt} was higher than daily
575 maximum air temperature in moist and wet tropical forest sites in Puerto Rico (Mau et
576 al. 2018). In the case of the *A. glandulosa* seedlings measured here, the mean
577 maximum daily temperature over the acclimation and measurement period was 40 –
578 42 °C (varying by treatment, Figure 1), matching the minimum potential T_{opt} of 40 °C,
579 and showing tolerance to the high temperatures to which they were exposed.
580 Measurement under higher temperatures would be necessary to find the T_{opt} for these
581 plants. Over the measured temperature range, g_s, V_{cmax} and J_{max} did not decline. Yet, it
582 should also be noted that the rates of A, V_{cmax} and J_{max} were fairly low (*c*. 3, 40 and 35
583 μmol m^{-2} s^{-1} respectively, at the highest values and under ambient CO_2). For example,
584 these are lower than A of 12 – 16 μmol m^{-2} s^{-1} across 42 Panamanian species (Slot &
585 Winter 2017c) and 5 – 12 μmol m^{-2} s^{-1} for four species in Puerto Rico (Mau et al.
586 2018), and V_{cmax} of 70 – 300 μmol m^{-2} s^{-1} and J_{max} of 80 – 220 μmol m^{-2} s^{-1} across four
587 Panamanian species (Slot & Winter 2017a), all at their optimum temperatures. The
588 measured rates are also lower than plants in other high temperature environments e.g.
589 five desert species with A ranging 19 – 35 μmol m^{-2} s^{-1} (Mooney et al. 1981), and
590 Mediterranean cork oak with V_{cmax} and J_{max} both over 150 μmol m^{-2} s^{-1} (Ghouil et al.
591 2003). Thus, high temperature tolerance of photosynthetic machinery in *A. glandulosa*
592 may come at a cost of lower photosynthetic rates. An alternative explanation for the
593 low photosynthetic rates is the low light conditions within the greenhouse, with
594 maximum leaf surface PAR of 800-1000 μmol m^{-2} s^{-1}. In the field, maximum PAR is
595 likely to be much higher (> 2000 μmol m^{-2} s^{-1}), and leaves may achieve higher
596 photosynthetic rates. The low light conditions in the greenhouse also have
597 implications for the high temperature tolerance observed. Because at high
598 temperatures photosynthetic biochemistry is under greater stress, there is a greater

need for photoprotection from high incoming radiation. Perhaps under the higher light conditions found in the field, very high temperature tolerance of photosynthesis may be more difficult to achieve. Field studies under high temperature conditions are needed to establish whether the high tolerance we find here also occurs under natural conditions.

As for g_s, the impacts of increased CO_2 followed expectations from previous studies with increased net photosynthesis when measured at growth CO_2, a steeper slope of A in response to temperature, and downregulation of photosynthetic capacity (Figure 8, 9). The steeper slope is due to the reduction in oxygenation of Rubisco due to higher c_i under elevated CO_2, which otherwise increases with temperature due to the reduced affinity of Rubisco for CO_2 with higher temperature (Long 1991). The effect of the downregulation can be seen when the temperature response of A is plotted with added points taken from the A-c_i curves at 400 and 800 ppm CO_2 for the elevated and ambient CO_2 treatments respectively, showing that without the downregulation of photosynthetic capacity A would have been higher in the elevated CO_2 treatment (Figure S9).

4.4 Conclusions

This study demonstrates that the tropical tree species *Alchornea glandulosa* shows strong responses of stomatal conductance to elevated temperature and of photosynthetic parameters to elevated CO_2. While a very high temperature tolerance of photosynthesis was observed in this species, photosynthetic rates were low under the high growth temperatures. These results show that this species will be able to cope

with the predicted atmospheric changes over the coming century. Therefore, it is an appropriate species for reforestation activities, which are planned and ongoing in the Atlantic forest (Rodrigues et al. 2009). More studies of other species are required to determine whether similar results occur in other forest trees.

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929

930 Tables

931 Table 1. ANOVA results for stomatal conductance linear mixed effects model.

932 Temperature and CO_2 refer to treatment effects. Asterisks denote *P* values: *** $P <$

933 0.0001, ** $P < 0.001$, * $P < 0.05$, • $P < 0.1$, ns not significant.

Model Term	Numerator DF	Denominator DF	F
Intercept	1	551	213.0***
Time	1	551	48.3***
$Time^2$	1	551	204.3***
PAR	1	551	28.5***
D	1	551	0.17^{ns}
Temperature	1	28	15.7**
CO_2	1	28	3.2•
Temperature:*D*	1	551	6.5*
CO_2:*D*	1	551	2.3^{ns}
Temperature:CO_2	1	28	1.3^{ns}
Temperature:CO_2:*D*	1	551	16.7**

934

935 Table 2. ANOVA results for A-T_L linear mixed effects model. CO_2 refers to treatment.

936 Asterisks denote *P* values: *** $P < 0.0001$, ** $P < 0.001$.

Model Term	Numerator DF	Denominator DF	F
Intercept	1	91	421.9***
Leaf Temperature	1	91	66.9***
CO_2	1	10	14.7*
Leaf Temperature:CO_2	1	91	11.8**

937

938 Table 3. Parameter estimates of Arrhenius functions of the temperature sensitivity of

939 V_{cmax} and J_{max}. Standard errors are given in brackets. Significance of between

940 treatment effects are shown: * $P < 0.05$, • $P < 0.1$, ns not significant. Letters denote

941 differences between treatments.

Chamber	$V_{cmax}25$	E_a (V_{cmax})	$J_{max}25$	E_a (J_{max})
aTaC	13.8 (2.74) AB	81702 (16778) AB	24.95 (1.14)	32187 (4279)
eTaC	14.2 (1.27) A	80644 (7551) A	24.0 (1.84)	27499 (7183)
aTeC	11.0 (1.75) AB	71004 (13689) AB	-	-
eTeC	10.5 (0.26) B	62050 (2171) B	21.8 (3.35)	25840 (14184)
Among Chambers	*	•	ns	ns

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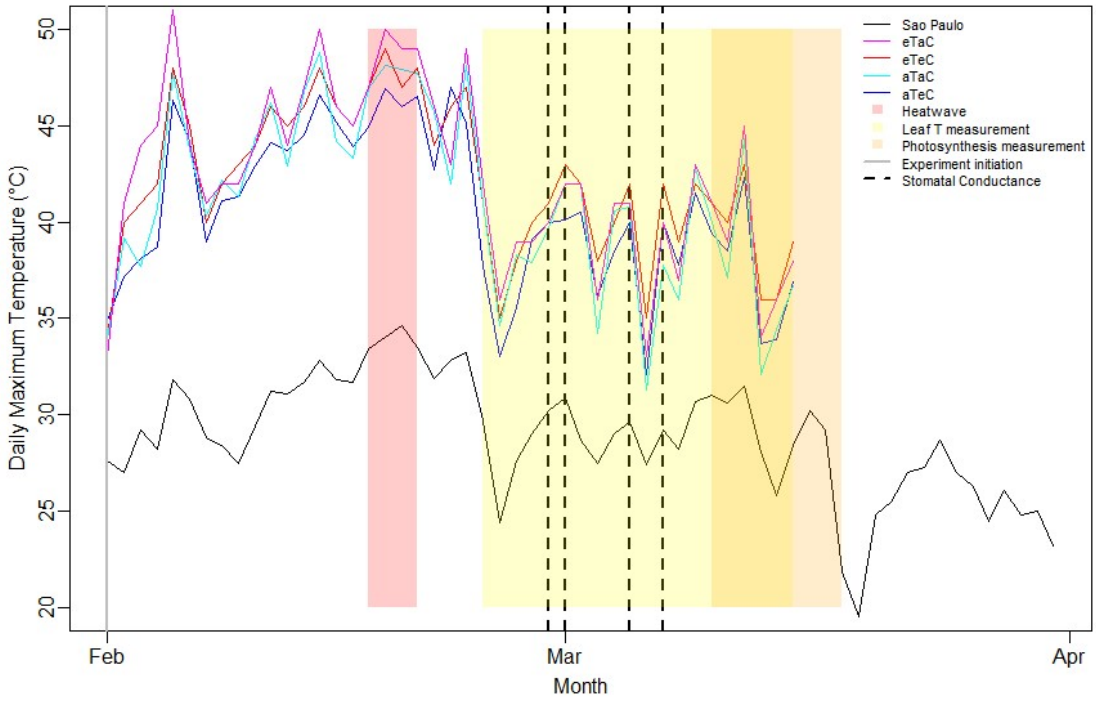
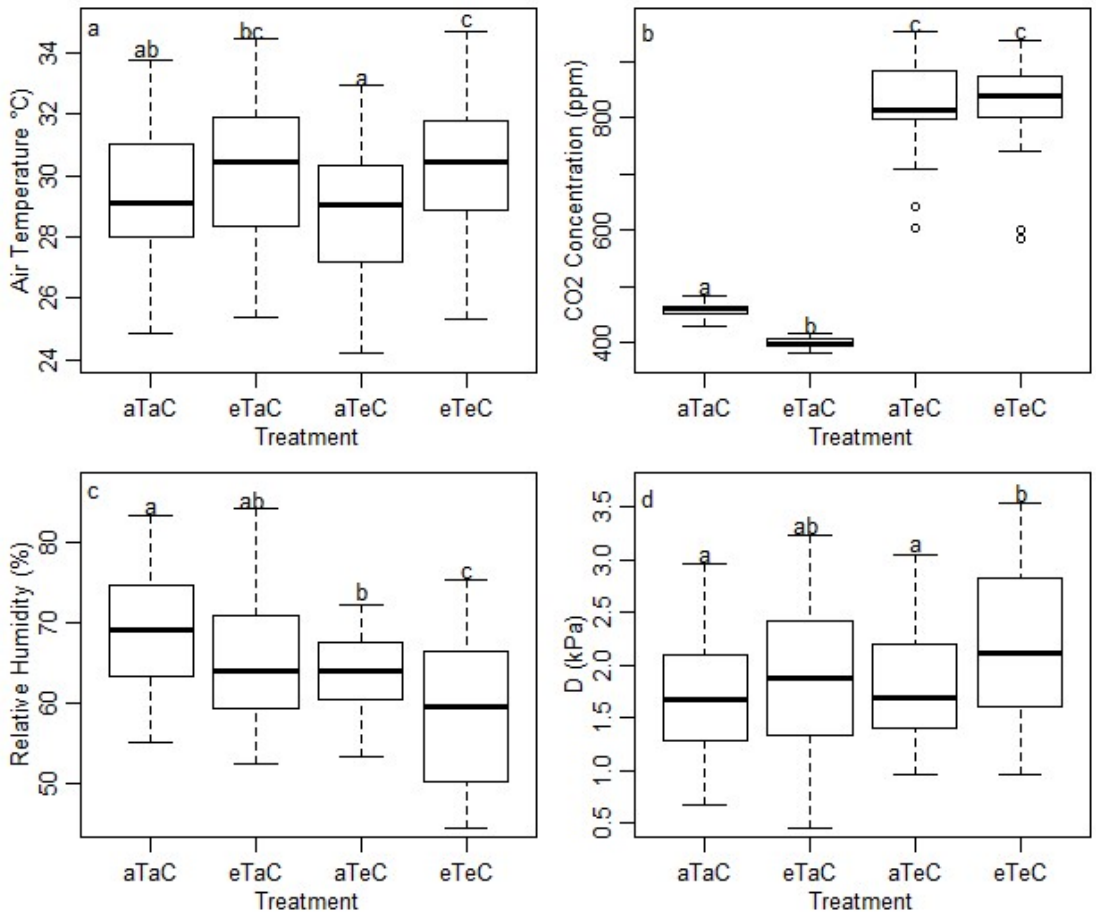
945 Figures



946

947 Figure 1. Time series of daily maximum temperatures in São Paulo (Mirante de
948 Santana weather station, data from INMET, http://www.inmet.gov.br/portal/, accessed
949 22/05/2018) and in each experimental chamber during the experiment. The
950 experiment was initiated on 1 Feb 2017. The period classified as a heatwave, periods
951 of leaf temperature and photosynthesis data collection, and days in which diurnal
952 cycles of stomatal conductance were performed are shown.

953



954

955 Figure 2. Differences in microclimate variables between chambers a) mean air
956 temperature, b) mean CO_2 concentration, c) mean relative humidity, d) mean *D*. Box
957 plots show daily averaged values from both the acclimation and measurement periods.
958 Treatments: aTaC – ambient temperature and CO_2, eTaC – elevated temperature and
959 ambient CO_2, aTeC – ambient temperature and elevated CO_2, eTeC – elevated
960 temperature and CO_2.

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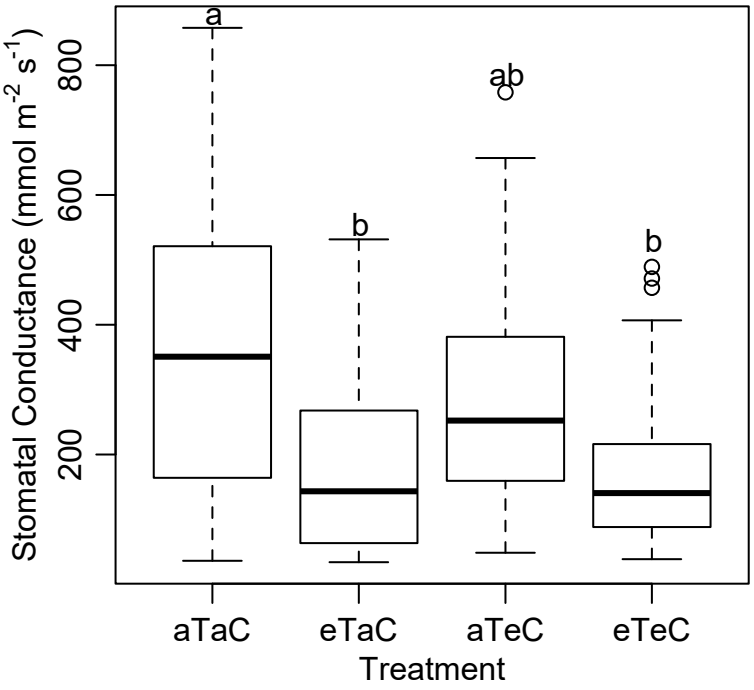

962

963 Figure 3. Effect of treatment on stomatal conductance where measurements from all

964 times of day are pooled. Treatments: aTaC – ambient temperature and CO_2, eTaC –

965 elevated temperature and ambient CO_2, aTeC – ambient temperature and elevated

966 CO_2, eTeC – elevated temperature and CO_2.

967

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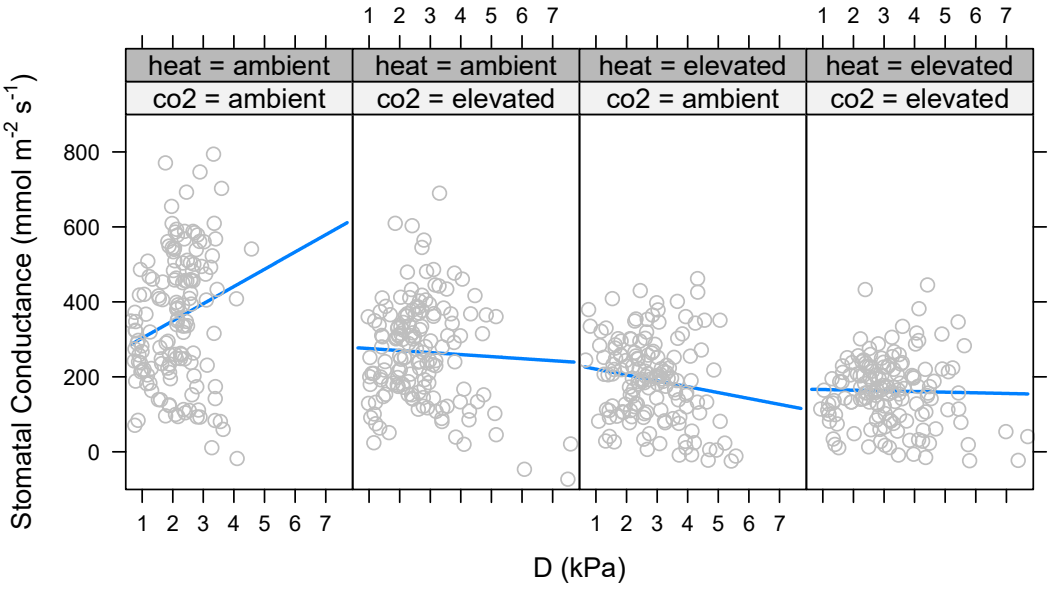


969

970 Figure 4. The relationship between g_s and leaf-to-air D for each treatment (accounting

971 for time of day (t, hours) and PAR as fixed effects and leaf as a random factor). Grey

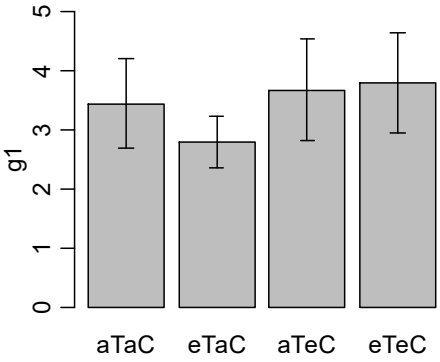
972 points show the partial residuals of the model. Full model equations: -1053.2 +

973 $215.6 \cdot t - 8.6 \cdot t^2 + 0.13 \cdot \text{PAR} + 45.9 \cdot D$ (aTaC); -1029.7 + $215.6 \cdot t - 8.6 \cdot t^2 + 0.13 \cdot \text{PAR} -$

974 $7.0 \cdot D$ (aTeC); -1074.1 + $215.6 \cdot t - 8.6 \cdot t^2 + 0.13 \cdot \text{PAR} - 17.3 \cdot D$ (eTaC); -1142.5 +

975 $215.6 \cdot t - 8.6 \cdot t^2 + 0.13 \cdot \text{PAR} - 2.6 \cdot D$ (eTeC).

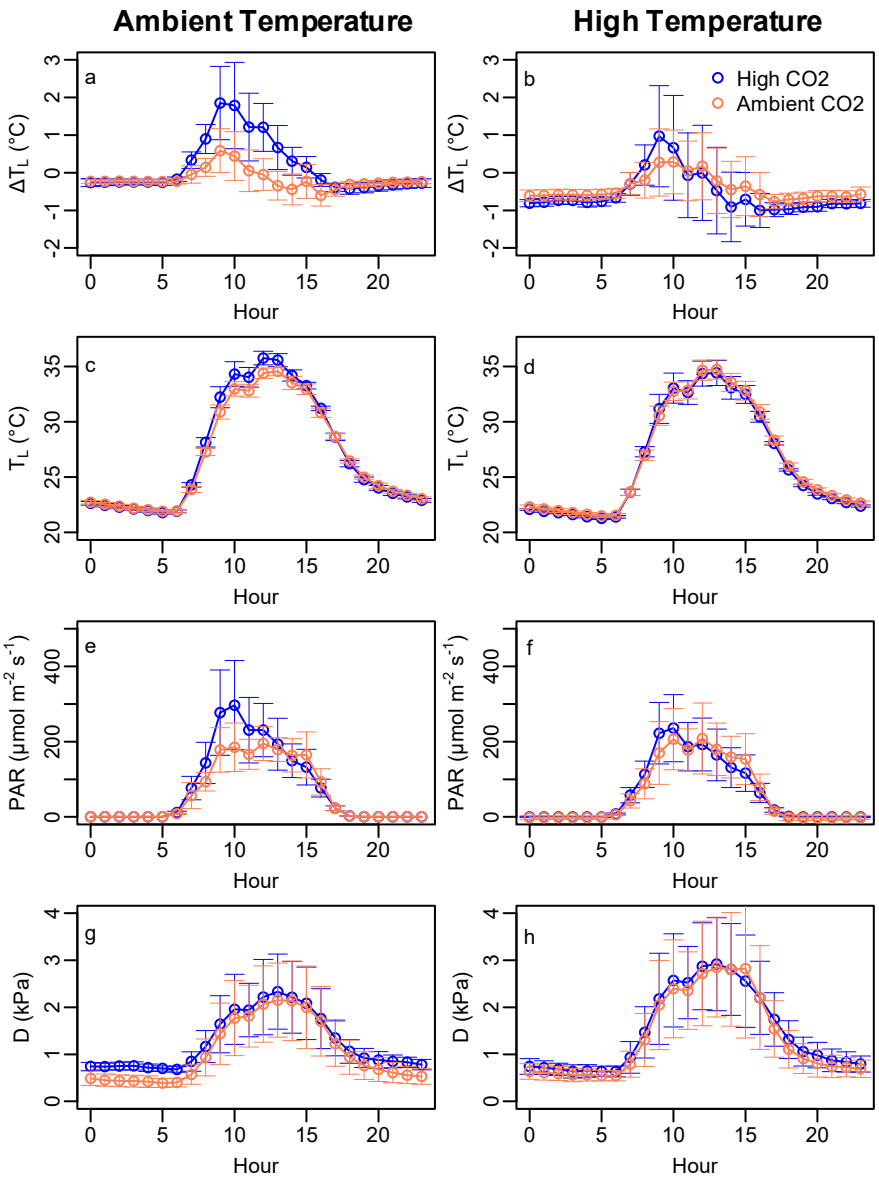
976



977

978 Figure 5. Comparison of the g_1 stomatal conductance parameter (unitless) between

979 chambers. Bars show the mean value and error bars the standard deviation.



980

981 Figure 6. Diurnal cycles of leaf-to-air temperature difference (a,b), leaf temperature

982 (c,d), PAR (e,f), *D* (g,h), for chambers with ambient air temperature (a,c,e,g) and

983 elevated temperatures (b,d,f,h).

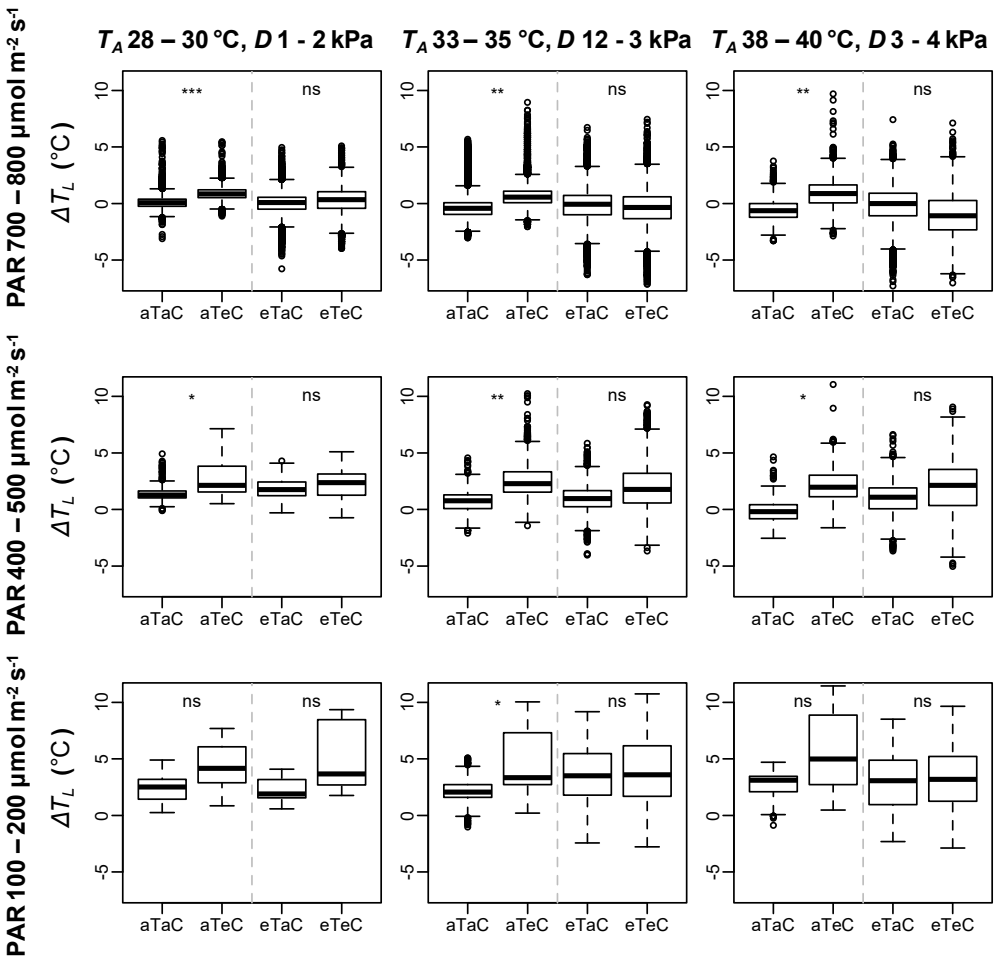
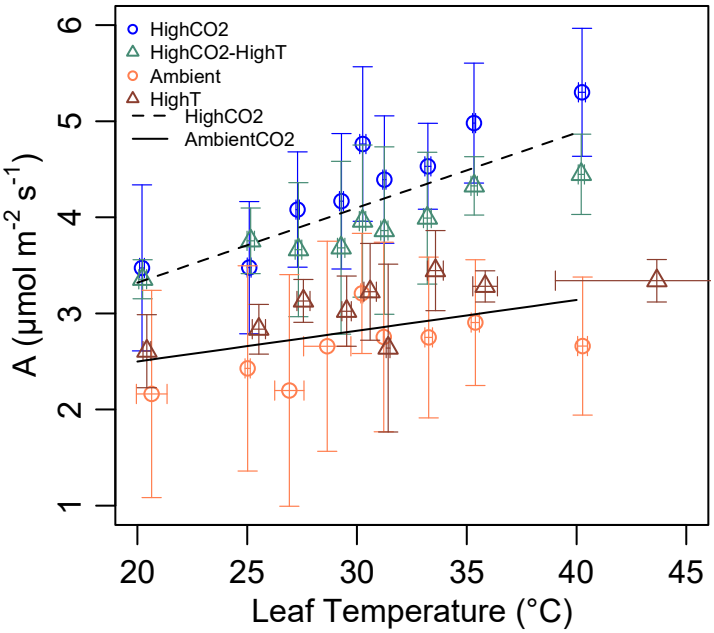


Figure 7. Leaf to air temperature differences for each treatment under a range of microclimate conditions. Contrasts are made between aC and eC under ambient the temperature treatment, and between aC and eC under the elevated temperature treatment, using mixed effects models with leaf as a random factor. Data is from ΔT_L measurements at 10 s temporal resolution subsetted for specific chamber air temperature (T_A) and D conditions, and leaf surface PAR conditions. Asterisks denote P values: *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$, ns not significant.



993

994 Figure 8. Temperature response of net photosynthesis. Under high CO_2 net

995 photosynthesis is higher and the temperature response in steeper. For ambient CO_2 A

996 = 1.86 + 0.032·T_L; for elevated CO_2 A = 1.75 + 0.078·T_L.

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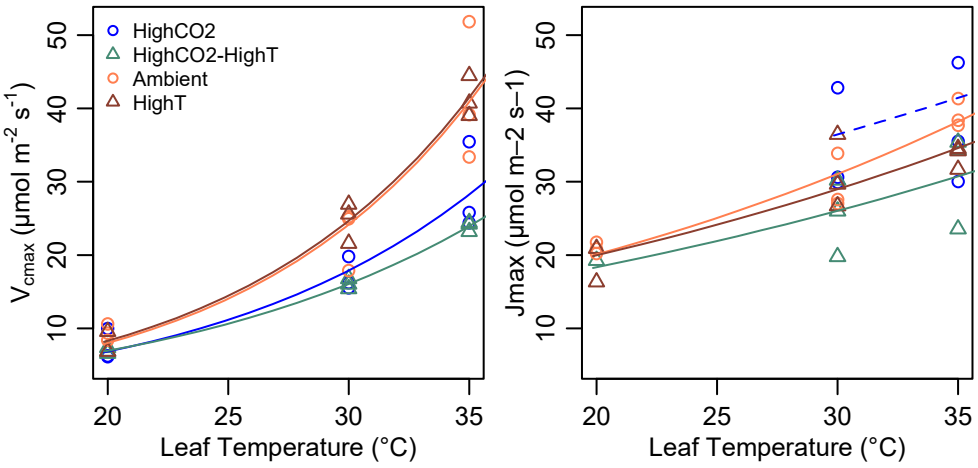

998

999 Figure 9. Temperature response of V_{cmax} and J_{max} fit with Arrhenius functions. For

1000 J_{max} in the high CO_2 treatment no values at 20 °C were obtainable. Equation

1001 parameters are given in Table 3.